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SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention should be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME V APRIL, 1930 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

RESEARCH ON THE CHEMISTRY OF DYES

One of the services that the Stain Commission has been able to perform for biologists is in assisting them to identify dyes of unknown composition with which desired staining effects have been secured. This enables them both to obtain new supplies of the same dyes and to publish their results in such a form that other biologists may duplicate them.

The analytical work involved in identifying these dyes has been done by the Color and Farm Wastes Division (the "Color Laboratory") of the Department of Agriculture, thanks to the coöperative arrangements that have been established with them. In the past, the amount of time they could give to such coöperation was decidedly limited, and it has never seemed wise to make any public announcement that such work could be done for individual biologists. At present, however, much more can be undertaken, because additional funds now supplied by the Chemical Foundation enable the Stain Commission to hire a research associate in chemistry (Miss A. R. Peterson) who is doing her work in the Color Laboratory and is able to test very promptly all the dyes the Commission needs to have analyzed, as well as to do some research work along similar lines. As a result the Commission is now equipped to do more of the work above referred to than ever before, and wishes to announce the fact to biologists in general. If any biologist has obtained valuable results with some dye, whose exact chemical nature he is not sure of, he is invited to refer the matter to the Commission chairman.

A further line of coöperation recently undertaken by the Color Laboratory is in connection with the Cancer Research recently establshed at the Johns Hopkins Hospital thanks to the generosity of Mr. F. P. Garvan and the Chemical Foundation. (See article by Geschickter, p. 49 of this issue). It was regarded very important in this work that dyes of exactly known chemical composition be secured. As this work was established in coöperation with the Stain Commission, it was easy to obtain the services of the Color Laboratory;

and in this way the Garvan Cancer Research Laboratory has been able to begin its investigations on a strictly scientific basis.

Fortunately the opportunities of the Color Laboratory for research on dyes are likely to increase rather than decrease in the near future; and the Stain Commission has been assured of their willingness to coöperate with biologists in the synthesis of dyes thought to have possible biological value. It is hoped, therefore, that any biologist interested in developing a new staining procedure or perfecting an old one will let the chairman of the Stain Commission know if he thinks some new dye might be of assistance to him.

H. J. Conn

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

Stains Certified November 15 to February 15, 1929*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Fuchsin, acid	NR 7	62%	As histological stain and for Andrade indicator	Nov. 21, 1929
Brilliant cresyl blue	NV 8	65%	As supravital blood stain	Nov. 21, 1929
Indigo carmin	NI 3	91%	As histological stain	Dec. 5, 1929
Crystal violet	CC 4	88%	As cytological and bacteriological stain	Dec. 19, 1929
Methyl green	NG 6	56%	As histological and bacteriological stain	Dec. 23, 1929
Hematoxylin	FH 8	——	As histological stain	Dec. 28, 1929
Methylene blue	CA 10	83%	As bacteriological stain	Jan. 24, 1930
Crystal violet	PC 3	78%	As cytological and bacteriological stain	Jan. 25, 1930
Tetrachrome stain	NMn 4		As blood stain	Jan. 28, 1930

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

THE HISTORY OF STAINING

The Development of Bacteriological Staining Methods

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The staining of bacteria is naturally of later origin than the use of dyes in histological work, as the systematic study of bacteria did not begin until after 1870. The use of dyes in this study followed very promptly after that date, however.

The beginnings of bacteriological staining were very adequately summed up by Loeffler (1887). The following quotation of about half of one of Loeffler's chapters (p. 213–220) is given here because it seems much more interesting than an original account of this early history would be if written today, nearly fifty years later.

"In his inaugural dissertation Weigert rightly laid stress on the positive diagnosis of bacteria as such and on the differences between them and other similar forms. For if one cannot distinguish the bacteria from other similarly shaped bodies, how is a closer study of these smallest beings possible? Weigert's service was to introduce into bacteriological investigations the staining methods already in use at that time in histological technic and to study them with this object in view. He, as a pathologist, succeeded especially in differentiating bacteria in the tissues from the other organized and unorganized particles present. After he had stained the bacteria in small-pox crusts with HCl-glycerin-carmin, this method and the hematoxylin procedure (first used by vonEberth in the study of bacteria, as pointed out by Weigert himself) were employed more and more by investigators for the same purpose—but only, indeed, with doubtful results. Wagner (1874) who made use of them in a case of intestinal mycosis, found both the hematoxylin and the carmin stain so unsatisfactory that he could attach no significance to them. 'Usually the threads are colored rather strongly' he wrote, 'but often they stain no better or even poorer than the rest of the tissue elements. I therefore ordinarily employed unstained preparations.' Weigert first succeeded, as we have seen, in so improving these staining methods as to make them practical. The methods still had various defects. Weigert, therefore, was unceasingly concerned to improve the old methods and to find better ones. On December 10, 1875 he described (Weigert 1876) before the Silesian Society of National Culture a case of ulceration at the navel also accompanied with compact white spots in the lungs the size of pinheads or millet seeds, and hemmorhages under the kidney and in

the neighborhood of the renal cavity. Weigert found the base of the umbilical abscess covered and the involved capillaries filled with very small, regular granules of sharp contour and presenting in general a chagreen appearance. The granules were insoluble in ordinary solvents. Alum-hematoxylin, however, colored them blue, as did methyl violet with subsequent washing of the preparation in dilute acetic acid; the mass became red with HCl-glycerin-carmin; brown with carmin and subsequent washing in dilute alcoholic liquor ferri sesquichlorati (complete nuclear staining). One can see the picture best by staining the preparation first with hematoxylin, then washing in dilute potassium hydroxide until it has a very weak blue color, treating further with strong acetic acid and finally examining in glycerin. It is often possible to stain only these masses blue and to see their grain clearly (with potassium hydroxide alone they lose their definition). The granules must for all these reasons be regarded as micrococci. In this contribution Weigert for the first time employed an anilin dye, methyl violet, for staining micrococci in sections of tissue. There followed next an accurate statement of his hematoxylin stain in a paper by Billroth and Ehrlich (1877). The directions run: 1.5 g. crystallized hematoxylin are dissolved in 50 g. alcohol of 0.830 sp. gr.; add 25 g. of a cold saturated alum solution and, after frequent shaking for 8 days, filter. Ten to 15 drops of this are placed in 25 g. distilled water or 0.5% alum solution, thus completing the staining fluid. The sections are left in this fluid for 3 to 4 hours, then rinsed with distilled water, and washed ½ to 1 hour in concentrated acetic acid until the acid remains colorless. Then they are carried over into distilled water, dehydrated in alcohol, cleared in turpentine and mounted in dammar. By this method Billroth and Ehrlich demonstrated micrococci in the edges of erysipilatous processes by means of sections of the tissue. At the Naturforscherversammlung at Munich, Weigert (1877a) showed sections of organs, spleen, lung, kidney and of dogs with anthrax, in which the anthrax organism could be distinctly seen in the tissue thru staining with methyl violet, gentiana, Bismarck brown, etc. and subsequent treatment with alcohol, etc.

"By these methods, the differentiation of micrococcus clumps and anthrax bacilli from tissue elements was assured. But how did the matter stand in regard to the recognition of bacteria in liquids?

"Hoffman (1869) had already made the observation about bacteria in his valuable treatise, that 'the little organisms were colored red after a while in aqueous solutions by means of coloring materials such as fuchsin solutions in water and acetic acid, as well as in carmin solutions, while the slime (in which they were embedded) remained

uncolored'; but he did not follow up these observations further. Salmonsen (1877) presented investigations concerning the staining of bacteria in liquids. After finding that iodine-potassium-iodide solution was a splendid agent for coloring bacteria in putrescent water but was not satisfactory for decomposing blood because it produced a finely granular precipitate in the latter, he discovered that rosanilin sulfate was an excellent medium for staining bacteria in putrescent blood. He used a concentrated aqueous solution which he prepared by boiling and filtered after cooling. He proceeded as follows: he put a drop of the putrefying blood under the cover glass in such a way that the drop did not reach the edge of the cover; then he let the staining fluid pass under from the edge and mix with the blood. Thus he obtained all possible shades of color. In this way he noticed that the weakly colored bacteria still moved while the intensively stained bacteria lost their motility, and moreover that the red blood corpuscles, thus disturbed, lost their color. In the investigation of old decomposed blood, the staining proved an especial advantage. In the unevenly granular bodies of such blood he saw the individual elements, at first wholly or partly unrecognizable, become easy to distinguish after the addition of the staining fluid. The anilin compound colored the protoplasm of the white blood corpuscles intensively red, gave a red tinge to the invisible stromata of the red corpuscles, left the fat crystals uncolored and made the various forms of bacteria stand out so clearly that they could be determined. The results which he secured were so good that he confined himself almost exclusively to staining with rosanilin sulfate in his investigations. This method of Salmonsen was not well known in detail, undoubtedly for the reason that the work was written in Danish.

"Another method of examining bacteria in fluids was published in the same year by Robert Koch (1877).

"Koch recognized that the chief difficulty in the examination of these small bodies without sharply visible edges lay in the fact that they either showed very active independent motility or else were in ceaselessly trembling molecular motion, and that even when at rest they were united in zoogloeal masses giving, on account of their low refractivity, the impression of a cloud-like picture, whose composition out of individual cocci or rods could not be recognized. Koch pointed out as an especial hindrance to bacteriological investigations the fact that there had been no method of preserving and making pictures of bacteria in their natural form and orientation (except when embedded in animal tissues) that were free from intentional or unintentional distortion. The drawings were usually to be regarded

as purely schematic and neglected the proportions so badly that it was impossible to use them for a comparison with reality. In accurate picturing of bacteria one can find only the slightest differences in size. Even tho a drawing of the largest bacteria might show exceptional care, it would remain questionable, nevertheless, whether the smallest forms could be so well drawn that the sketches would correspond with the originals and could not be confused with similar forms.

"Koch was able to avoid all these difficulties in a brilliant manner. His new procedure consisted 'of drying the bacterial suspension in a very thin layer on the cover glass in order to fix the bacteria in one plane, of treating this smear with stains and washing, in order to return the bacteria to their natural form and to make them clearly visible, so that the preparation thus obtained could be mounted in preserving fluids and finally be photographed for obtaining accurate pictures'. Koch accomplished the drying by taking a small drop of the fluid under investigation—putrefying blood, tooth scrapings, or putrefying infusions—on a scalpel and spreading it with a circular motion in the thinnest possible layer, so that the bacteria, blood corpuscles, etc., were not piled up but were separated from each other by larger or smaller spaces. To his surprise he saw that the bacteria adhered to the glass like stiff bodies surrounded by a mucous membrane and dried without appreciably changing their shape either in length or width. The only changes occurring which he could observe consisted in the flattening of spherical, lobate, or branched zooloeal masses and in the conversion of spiral bodies into wavy lines. On the addition, however, of a drop of potassium acetate solution (1 part to 2 parts distilled water), the mucous sheath of the bacteria could be made to swell up again so that the zoogloea and the spirals resumed their natural form. He showed by means of experiments in returning the dried preparations to water or glycerin that the smear could be quickly loosened and floated off the glass. By immersing the cover glass in absolute alcohol or even better 0.5% chromic acid, the smear could be made almost impossible to remove; but the bacteria, whose mucous sheathes could no longer swell up, could not resume their natural shape. The only practical means, which also served as a splendid preservative, was the solution of potassium acetate. The smallest forms became thereby so pale that it seemed necessary to make them more distinct by means of dyes.

"Koch investigated for this purpose the most varied dyes used in dyeing and in microscopy, in order to determine their behavior toward bacteria, and determined that of all dyes the anilin colors were best suited for staining bacteria, because the bacteria 'take up the anilin dyes with so much certainty, so quickly and so deeply

that one can use these dyes as reagents for the differentiation of bacteria from crystalline and amorphous precipitates as well as from small fat drops and other minute bodies'. The anilin dyes showed the valuable property of merely moistening the dried smear without loosening it. The water-soluble anilin dyes proved the most useful, particularly methyl violet (BBBBB) and fuschin, while the others, namely safranin, yellow, eosin, orange, methyl green, iodine green, and blue, did not stain so strongly and proved to be unstable. To stain the dried preparation on the cover glass, Koch added a few drops of a concentrated alcoholic solution of the anilin dye to 20 to 30 g. of distilled water. He placed a few drops of this on the bacterial smear to be stained and let it stay in contact for a few seconds with continual motion. Then he blotted off the staining fluid with filter paper or else washed it off either with distilled water or with a 10% solution of potassium acetate. The stained preparations were then mounted for preservation either moist in potassium acetate (1:2) or dry in Canada balsam.

"In order to photograph bacterial preparations, Koch turned his attention to the anilin dyes which did not transmit the chemically active light rays, in other words the blue part of the spectrum. Anilin brown gave him the best results. He dissolved the anilin brown in equal parts of glycerin and water, covered the preparation a few minutes with the fluid, washed it off with pure glycerin and mounted it in glycerin. Certain albuminous substances, such as blood, pus, etc., which were difficult to stain with watery solutions of methyl violet and fuchsin, gave splendid preparations with the brown dissolved in glycerin. . . .

"By means of a special procedure—treating the cover-glass preparation with concentrated aqueous solution of logwood extract and subsequent application of weak chromic acid solution or Müller's fluid—Koch succeeded in staining flagella, the bacterial organs of locomotion, whose staining, all students including von Billroth, had been striving for in vain. By means of his clear photographs of these he was able in one stroke to confirm beyond question the existence of these structures which had been seen by Ehrenberg on his *Bacterium triloculare*, by Cohn on *Spirillum volutans*, by Dallinger and Drysdale on *Bacterium termo*, and by von Warming on red chromogenic vibrios and spirilli, but whose reality was questioned by various others."

This historical discussion of Loeffler's brings the matter up to the year 1878. It was published nearly ten years later, and as the book was designated "Erster Theil" it is assumed that its author contemplated a later publication of some of his other lectures in which

he brought the subject up to the date of writing. No such publication followed, however, and as a result he never included some of the very important papers on the subject appearing during the eighties.

Ehrlich (1881) was certainly the first investigator to employ methylene blue as a bacterial stain, and possibly the first to use this dye in any line of microscopic work. He stated that for bacterial investigation only the basic dyes were satisfactory, but that the ordinary dyes such as Bismarck brown, fuchsin and methyl or gentian violet stained bacteria too intensively, some forming a granular precipitate which was easy to confuse with the bacteria. Ehrlich found methylene blue free from this disadvantage. He employed a saturated aqueous solution and allowed the dried preparation to stand in it from one-half hour to twenty-four hours followed by washing and mounting in Canada balsam.

The following two years saw the principal advances in the staining of bacteria applied to the study of the tubercle organism. Koch (1882) had just begun his classic contributions to the knowledge of this disease and everyone was much interested in staining and investigating the organism. Koch himself stained this organism in tissue with an alkaline methylene blue solution, followed by a concentrated aqueous solution of Vesuvin (undoubtedly Bismarck brown) as a counterstain. His alkaline methylene blue consisted of 10 parts of concentrated alcoholic methylene blue to 0.2 parts of 10% potassium hydroxide.

The same year Ehrlich (1882) introduced the principle of adding anilin water to the solution of a basic dye. He assumed that the action of Koch's alkaline methylene blue was due to the fact that the tubercle organism possessed a membrane that could be penetrated only by alkali. He regarded anilin oil as a satisfactory substitute for potassium hydroxide in this respect. In employing it he mixed about 5 cc. of anilin with 100 cc. of water, and shook the mixture and allowed it to stand a while. It was then filtered thru a wet filter paper. Of a concentrated alcoholic methyl violet solution, 11 cc. was added to 100 cc. of this anilin water; or enough concentrated alcoholic fuchsin was added to the anilin water to cause a metallic film on the surface. Ehrlich observed that preparations thus stained could be treated with strong hydrochloric acid solutions without the tubercle organisms losing their color, while the tissue and other bacteria was decolorized. A similar observation had been made concerning the leprosy organism by Neisser (1881) the preceding year. The diagnostic value of acid-fastness, however, does not seem to have been appreciated at this time.

A further simplification was introduced a little later in the same year by Ziehl (1882) whose staining solutions are more generally employed today on account of their better keeping qualities. He found that not all samples of anilin oil worked satisfactorily in the Ehrlich procedure and concluded that the behavior of the satisfactory lots might be due to impurities in them. He therefore tried phenol in place of anilin water in his methyl violet solution, and found it very efficient. Hence he opposed the conclusion of Ehrlich that the alkaline reaction of the staining fluid was necessary for the demonstration of the tubercle organism.[1] Resorcin and pyrogallic acid acted equally favorably.

The staining solution of Ziehl is now ordinarily employed in the Neelsen (1883) modification—carbol-fuchsin instead of carbol-methyl-violet. Neelsen prepared the staining fluid by adding 10 cc. of a concentrated solution of basic fuchsin in absolute alcohol to 100 cc. of 5% phenol solution. Bacteriologists often today speak of Ziehl's carbol-fuchsin; it is interesting to notice that this particular stain originated with Neelsen rather than with Ziehl. Later modifications of the procedure consisted primarily in changes in the method of decolorizing. Important among them is that of Gabbet (1887) in which the decolorization and counterstaining were carried on simultaneously in a 1–2% methylene blue solution in 25% sulfuric acid.

At about this same period Loeffler (1884) proposed his alkaline methylene blue which is still in common use. The idea of an alkaline methylene blue, as shown above, originated with Koch; Loeffler's contribution was to use a more dilute solution of potassium hydroxide. His formula called for 30 cc. of saturated alcoholic methylene blue to 100 cc. of 0.01% potassium hydroxide. The addition of this weak alkali undoubtedly has the advantage of allowing the dye to stain equally intensely without causing its solutions to polychrome too rapidly on standing. For this reason, probably, the Loeffler formula was the one to come into common use for general bacterial staining and especially for staining the diphtheria organism, as recommended by its author. (With modern methylene blues, it should be added, an alkaline formula seems to be no longer necessary).

The same year saw the first publication of the famous Gram (1884) stain. His original method called for the use of anilin gentian violet, which Gram calls Ehrlich's, altho his formula was slightly different

[1]It is perhaps of interest to note here that modern investigations show Ehrlich to have been entirely right as to the greater staining power of basic dyes in alkaline solution. Ziehl by adding such chemicals as phenol and resorcinol was actually employing new compounds which stain more intensely than the simple dyes. (See Holmes and Hann, 1928).

from the one given above (p. 44). This solution was kept in contact with the preparation from one to three minutes and then Lugol's iodine solution (iodine 1 part, potassium iodide 2 parts, distilled water 300 parts) was applied for a similar length of time. Decolorization was effected with absolute alcohol applied for 30 seconds, sometimes substituting clove oil in the latter stages of the differentiation, especially when the organisms were studied in the tissues. In staining sections, the method was somewhat altered, with other basic dyes and other modifications of the iodine. It is to be noticed that Gram did not use any counterstain. Neither did he appreciate the significance of the fact that some bacteria did not stain by this method.

The idea of Gram-positive and Gram-negative bacteria was a later introduction; so was the principle of using a counterstain for the negative organisms. As a matter of fact the technic has been modified and remodified so many times that as carried on in the usual modern laboratory it bears little resemblance to the original procedure of Gram's. The most common modifications consist of the use of safranin, pyronin, eosin, Bismarck brown or some other dye as a counterstain, and the substitution of some other mordant, such as phenol or ammonium oxalate, for the anilin water. Especially important was the use by Weigert (1887) of the Gram technic following carmin as a tissue stain, decolorizing with anilin instead of alcohol. In some of the very recent modifications acetone is the decolorizer.

Had Loeffler ever finished his history of bacteriology he would have had one very important contribution to the staining of bacteria of his own to record. His papers on flagella staining (1889, 1890) not only proposed a practical method of staining these delicate structures, which is still used with only minor modifications in many modern procedures, but also contributed much of theoretical interest concerning staining methods. He accidentally ran onto the principle of mordanting in the study of the capsules of pneumococci. In this work he used a gallnut ink; and when he subsequently stained with methylene blue was surprised to find that the capsules as well as the cells were colored blue. This suggested that the ink had had a mordanting action and he wondered if it could not be similarly employed to facilitate the staining of flagella. He tried it with a moderate degree of success; and then to investigate the principle and to improve the technic, he began trying various synthetic inks. The compound obtained by adding ferrous sulfate to tannic acid was among the best; so was a boiled extract of logwood. The first mordant which he proposed (1889) called for the addition of logwood extract to the iron tannic acid compound. In his later paper (1890) he modified this by substituting an anilin basic dye for the logwood extract

(usually fuchsin or methyl violet). Of even greater importance, however, was his investigation in this second paper of the influence of reaction upon the staining. Reaction in its relation to H-ion concentration was not then understood, but Loeffler showed how to obtain a series of mordants varying in reaction by adding a certain number of drops of either 0.25 N sulfuric acid or 0.25 N (i.e. 1%) sodium hydroxide. He further showed that different kinds of bacteria varied in the reaction of the mordant necessary to secure the most successful staining of the flagella. In some instances he added as much as 9 drops of the acid, in others as much as 36 to 37 drops of the alkali; while with at least one organism he found best results with no adjustment of the mordant. He thus showed that flagella staining was no simple matter, a fact which bacteriologists still realize today. Recent literature is full of efforts to obtain a single universally satisfactory procedure; a careful reading of Loeffler's second paper on the subject, however, shows perhaps why such modern efforts do not meet with a high degree of success.

The last three decades have witnessed several studies of the internal structure of bacteria. It has always been assumed that bacteria are single celled organisms, and biologists have naturally been interested to see if the ordinary details of cell structure could be demonstrated in them. In attempts to answer this question a great variety of staining methods have been devised, the most common principle underlying which has been the use of dilute solutions of the basic dyes, sometimes applied to dried films of the bacteria, sometimes to the living cells without drying. The results of such studies have sometimes been conflicting, or at the best have led to no very definite conceptions as yet concerning bacterial morphology. For this reason it is still too early to evaluate the methods and to decide which are the outstanding procedures. The single exception to this statement perhaps is the work of Arthur Meyer and his students, (see Meyer, 1903) who were able by means of dilute methylene blue, dilute fuchsin and iodine to demonstrate three types of granules in the spore-forming bacteria which Meyer regarded as reserve foodstuff; two of these types of granules he considered to be fat and glycogen respectively, while the third which was of unknown nature he named volutin.

Present investigations concerning staining methods are very largely along this latter line, that is, efforts to devise methods capable of telling more about the internal structure of bacteria. Considerable work is also in progress to learn the interpretation of certain staining reactions, especially that of the Gram stain. This has become very interesting to bacteriologists because of its apparent relation to the inhibition of bacterial growth by means of dyes.

Meanwhile parasites other than true bacteria have been discovered, such as Treponema, spirochaetes, and protozoa; and the ordinary bacterial stains have rarely proved suitable for demonstrating these organisms. Methods have been devised for this purpose so different from these discussed above that they will not be considered here but will be taken up in a later paper.

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THE APPLICATION OF DYES IN THE CANCER PROBLEM

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ABSTRACT—**Three fundamental requirements for the problem of developing a differential stain for cancer are discussed: I. the choice of a technic for the microscopic preparation of tissues; II. an analysis of the biological properties peculiar to cancer; and III. various groups of dyes adaptable to such peculiar properties of cancer tissue. Under I the disadvantages of intravitam staining are pointed out and the use of cell suspensions, frozen sections, and fixed material favored. Under II three characteristics of cancer tissue offering possibilities for differential staining are discussed, the cytological structure known as the "plastin reaction", the histogenic cycle of cancer tissue, and the viability of cancer tissue under anaerobic conditions. Under III modifications of the Giemsa stain are suggested for application to the plastin reaction, specific tissue stains advocated for the use of indicating end points in histogenic cycles, and the vital dyes, congo red and trypan blue, suggested as indicators for the survival of malignant tissues because of the failure of these dyes to permeate living cancer cells.**

The angle of approach thruout has been an attempt to avoid unconscious pitfalls inherent in certain microscopic technics, and to substitute analytical methods for the blind trial and error method of routinely applying dye after dye in endless succession.

INTRODUCTION

The purpose of seeking a differential stain for cancer is to supply a technic for the rapid visual recognition of cancer tissue which will be available and reliable in the hands of all those clinicians having occasion and need to resort to a differential diagnosis between benign and malignant conditions.

Three fundamental assumptions are employed in the resort to dyes in this problem.

First: That there are reactions, biological, physical or chemical, peculiar to the cancer cell which are not found in other diseased conditions or normal tissues.

Second: That there are certain dyes which bring about a color change in the tissues when brought into contact with such reactions, which will serve as visual indicators for these reactions.

Third: That biopsy is feasible as a diagnostic procedure and that this method will make possible the gross or rapid microscopic exami-

nation of the reaction of the excised (or incised) cancer tissue with an appropriate stain.

Some unique property of the cancer cell, a proper dye to serve as indicator, and a procedure for tissue examination are therefore the three fundamentals of the problem of differential staining in cancer. The probability of meeting these three requirements successfully is within the bounds of established facts.

In regard to the first, reactions peculiar to the cancer cell, it has long been known that cancer cells not only grow at a different rate and in a different manner from other tissues, but that these cells also show peculiar chemical reactions such as increased acidity[1] with an increase in lactic acid production[2] and decreased glycogen content[3], with growth under abnormal oxygen tensions[2] and a high electrical conductivity[4]. More recently Lipschütz[5] has shown that the internal cell structure differs from the normal cell by his so-called "Plastin reaction" and more grossly Geschickter[6] in a forth coming article on Osteogenic Sarcoma has shown that both the individual cancer cells, as well as the generations of daughter cells complete a life cycle peculiar to malignancy but allied to embryonic tissues. Thus there are available in the cells of cancer tissue various factors, which if capable of measurement and rapid demonstration, may serve as a basis for the development of a differential staining reaction.

In regard to the second requirement, the use of dyes as indicators, it has been well established by a long series of brilliant investigators such as Ehrlich[7] working with indigo, Ehrlich[8] and Koch[9] working with methylene blue, and more recently by W. M. Clark[10] and co-workers using these dyes and the indophenols and meriquinones—

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[1]Carrel, Alexis. Tissue Culture and Cell Physiology. Phys. Rev., 4, 1. 1924.

[2]Warburg, Otto. Ueber den Stoffwechsel den Tumoren, Springer. Berlin. 1928.

[3]Bierich, R. and Rosenbohm, A. Untersuchungen uber die Biochemie der Drebsbildung. Biochem. Zts., 152; 193. 1924.

[4]Crile, G. W. A Bipolar theory of Living Processes. Macmillan. New York. 1926. See p. 112–129.

[5]Lipschütz, B. Ergebnisse Cytologischer Untersuchungen an Geschwielsten. Zts. für Krebsforschung. 28, 491. 1929.

[6]Geschickter, C. F. Osteogenic Sarcoma. Arch. Surg. To be published.

[7]Ehrlich, P. Antikritsche Bemerkungen über Drüsenfunctionen. Centr. med. Wis. 23, 161. 1885.

[8]Ehrlich, P. Methylenblau-reaktion der lebenden Nervensubstanz. Deut. med Wochenschr. 12, 49. 1886.

[9]Koch, A. Über den schwefelhaltigen Farbstoff aus Dimethylphenylen diamin. Ber. 12, 592. 1879.

[10]Clark, W. M. Studies on Oxidation-Reduction. Hygienic Laboratory Bul. 151, 1. 1928.

that dyes are excellently suitable for the demonstration and measurement of slight changes in acidity content, oxidation-reduction intensities and other similar reactions such as those involved in cancer tissue. More specifically, Lipschütz, working with fixed cancer tissue has shown the direct applicability of the wet Giemsa stain to peculiarities of the malignant cell. The practical application of a differential stain for the rapid visual recognition of malignancy in the hands of clinicians does not therefore seem to be a remote or chimerical possibility.

In reference to the third requirement, a method of tissue examination, both the value and technic of biopsy have been well established. From an operative viewpoint it has been conclusively shown by Bloodgood,[11] MacCarty[12] and others, that a decision reached by biopsy at the time of operation and immediately acted upon, eliminates any danger from impairment of the therapeutic results by the dissemination of tumor cells at the time of biopsy. Also from the standpoint of judging the results of therapeutic methods in cancer, a preliminary biopsy to substantiate the diagnosis and to grade the degree of malignancy present is almost indispensible to the reliable evaluation of any new mode of cancer treatment proposed. Biopsy, of course, since the changes in cancer tissue are of a microscopic order, must include methods for the fixation and microscopic examination of tissues, and fortunately among the various procedures well established, several rapid methods such as the frozen section technic, are available. But from the standpoint of differential staining only those methods for the hardening and microscopic preparation of tissues are acceptable, which do not introduce an extraneous group of reactions, which will either negate or obscure the type of changes that we are endeavoring to study. Some microscopic technic, adaptable to biopsy, and which will not obscure the most delicate reactions of the cancer cell, should be available for differential staining.

Thus in devising a stain, for the differential diagnosis of cancer we must analyze in detail (1) the peculiarities of the cancer cell, (2) the properties of the indicator dyes, and (3) the various technics for the microscopic preparation of tissues.

I. Technic of Tissue Preparation for Microscopic Work

Because of their bearing upon both a study of the properties of tumor cells, and the biological properties of stains it is best to con-

[11]Bloodgood, J. C. Tissue diagnosis in the operating room. Southern Med. J. 21, 179. 1928.

[12]MacCarty, W. C. The cancer cell in general practice. Surg., Gynec., and Obstet. 47, 566. 1928.

sider first the various methods of preparing tissue at our disposal for microscopic and experimental work. Broadly speaking, three different methods are available: first, examination of tissues in vivo by tissue culture and the hanging drop technic; second, frozen section or teasing of fresh tissues for immediate examination; and third, permanent fixation of the material. All three of these technics have their special applications, their peculiar advantages and disadvantages, and it is well to keep these definitely in mind.

A

The examination of tissues in vivo has certain advantages in the study of the properties of the cancer cell. Cancer cells in tissue culture, or immediately after removal of the tissue from the tumor proper in physiological solutions, present the most favorable conditions for the study of various features of the biochemistry of tumor cells, altho even under such conditions it is important to realize that, strictly speaking, there are no true physiological solutions outside of the animal body; and even minute traces of copper in the water (often present as a result of the coils used in distillation) as Bierich has shown, may be sufficient to obscure important cellular reactions. The living tumor in situ may even be subjected to intravitam staining, but this method, while important from the standpoint of the therapeutic application of dyes, like all of the "in vivo" methods of tumor study, presents many serious disadvantages from the angle of the biological study of stains.

In such a biological test of various dyes we are usually seeking to bring about a reaction between some unique property of the cancer cell and some chemical property of the stain, assuming the former to be constant, and varying the latter by trying in succession a variety of stains. As a matter of fact we are dealing with a far greater range of variables than is implied in any such simple experimental maneuver. In all of the "in vivo" methods this variability is tremendously increased.

In the first place the dye is subjected to a group of influences which may entirely conceal its chemical properties. In intravitam staining the blood serum and the reticulo-endothelial system of the animal or patient so combine or react with the dye as to remove practically all of it from contact with the cancer cell. What dye may reach the proximity of the tumor is kept from reaction by the cell membrane which practically excludes all of the ordinary dyes from the interior of the cell. In addition, the whole of the tumor being present in the living animal and a large portion of the tumor invari-

ably consisting of necrotic or dying tumor cells, these cells with their injured cell membrane will take up the dye rather than the sound cells, and thus increase the improbability of the dye ever reaching the active tumor cells.

Thus the intravitam method of staining, altho providing the active cancer cell under practically ideal condition, presents a maximum of adverse conditions for the dye. The concentration of the dye must be small at the time of injection to avoid killing the animal, and this concentration is successively depleted by the blood serum, the reticulo-endothelial system, necrotic tumor cells, and the cell membrane of the living tumor cells. Granting that even under such conditions, dye did reach the interior of active tumor cells, to substantiate the finding, microscopic examination must be carried out, and in preparing the tissue for the microscope the reaction might be hopelessly obscured.

Using the other vital methods of supravital staining of tissue cultures or tumor tissue in physiological solution, we have similar disadvantages. The intact cell membrane is still a barrier to the dye, and the culture medium with its serum, or the physiological solution with its salts, is first to come in contact with the dye and is apt to alter its reactions seriously. Here again the concentration of the dye must be limited to avoid injuring the finer cellular structures. The two outstanding advantages of the supravital staining technic are the living state of the tumor cells, and the fact that these cells may be observed directly, without fixation, under the microscope. The counterbalancing disadvantages are the intact cell membrane, the culture medium and the physiological solution, and the limited concentration of the dye. Of all these factors, the impermeability of the intact cell membrane, to most dyes, is outstanding.

In weighing the various vital methods of staining for use in the laboratory, we can rule out the method of intravitam staining as being hopelessly cumbersome for experimental purposes, and because it is not available for use on human tumors. The supravital staining technics are more desirable, but impose the handicap of an intact cell membrane in the tumor cells.

B

The employment of frozen sections in the investigation of the staining reactions of cancer tissue approaches the technic of supravital staining. Here we have not the handicap of an intact cell membrane, and the tissue is more suitable for microscopic examina-

tion. The outstanding disadvantage of this technic is the time limitation imposed. From the moment of freezing (which must be carried out immediately on the operative specimen) degenerative changes begin to make their appearances in the cell, and the staining reaction must, therefore, be immediately subjected to microscopic examination. Hence the method is more suitable for practical application in the operating room than experimental studies in the laboratory. By combining the frozen section method, however, with the use of some physiological solution for maintaining the viability of operative specimens, we have a technic which offers definite possibilities.

C

Because of the instantaneous fixing of the tissues, and the permanency and convenience in handling the material for study, carefully prepared sections of fixed and hardened tissues constitute one of the best of the microscopic methods for the study of tumor tissues. Here again there is danger of introducing factors which will obscure important features of both the dye and the tumor cell, and in order to overcome these possible disadvantages, modifications in the usual routine technic must be instituted.

First, preservation of the living cell structure to a maximal degree requires immediate fixation of the tumor tissue at the time of its operative removal. Material from the autopsy room is absolutely unusable, and operative specimens carried unfixed from the operating room to the laboratory are also undesirable. When proper fixation is carried out at the operating room, many of the reactions of the living cell can be better studied in hardened preparations than on living cells in physiological solution, as demonstrated for lactic acid production by Bierich.

However, the advantages of immediate fixation may be lost if the chemical nature of the fixing fluid is not carefully controlled. Fixing fluids which act either as strong reducing or oxidizing agents, or which have a decided basic or acid character, are capable of so altering the chemical reactions of the cancer cells that the very changes which are essential to the success of staining are hopelessly altered before the tissue reaches microscopic examination. Among the large variety of fixing fluids that have accumulated thru the collective work of many investigators, the vast majority are unsuitable for staining investigations in cancer. A few of these solutions, like Regaud's (which balances the oxidizing effects of potassium dichromate against the reducing action of formalin) or Schaudin's (which balances the basic reaction of alcohol against the acid salt

of mercury), are suitable for use when the proper chemical standardization is employed. The fixing fluid must be kept at the neutrality point in regard to such important relations as oxidation-reduction and acid-base equilibrium, if the reaction of dye to tumor cell is not to be obscured.

Once properly fixed, the material used for embedding the tissue, as well as any salts deposited in the tumor by the fixing fluid, may be removed by suitable solvents after the tissue has been sectioned.

When a carefully controlled technic is used, tumor tissue which is fixed and embedded, permits sections of an almost unlimited number from a single tumor specimen, and the staining reactions of the tumor may be studied over an indefinite period of days or months. Such a flexible reservoir of material for investigative purposes is not available by the other methods. With such a microscopic technic there is no progressive degeneration of the cell structure, as in frozen sections, no impermeable cell membrane to combat the dye, and the concentration of the dye need not be adapted to physiological conditions as in intravitam or supravital staining. It is true that staining reactions demonstrated for fixed tissues are not readily adaptable as therapeutic agents and may even present handicaps in rapid diagnostic work at biopsy. But these practical considerations are of secondary importance only, from the scientific standpoint of finding a unique reaction between the cancer cell and a differential dye, and only serve to complicate the search at the outset. They are best handled as corollary problems in applying the findings arrived at by other procedures.

In reviewing the various microscopic technics for tissue study, therefore, it is evident that all show certain disadvantages for the problem in hand. Some of these disadvantages are serious obstacles to investigation, such as those encountered in intravitam staining and in supravital methods; while the frozen section and methods of permanent fixation of material are capable of modifications which adapt them to a study of the biological reactions of dyes. We have seen however that the "in vivo" methods are important for the study of the properties of the cancer cell, and as such there is occasion to employ them.

It is important to emphasize here, that whatever the methods of tissue preparation employed, if care is not exercised in selecting the specimen of tissue from the tumor, important reactions of the cancer cell may be obscured by changes occurring in the living tumor such as central necrosis, secondary infection, hemorrhage from operative manipulations, etc. Numerous investigations have established

the fact that the malignant cell is short lived, and many of the cells pass rapidly into degenerative changes. It is only at the tumor periphery that the malignant cell is functioning as such, and it is here that the most favorable specimens for study are obtained. It must be borne in mind that even here, there is an admixture of tumor cells with normal reacting tissue.

II. The Specific Property of the Malignant Cell

No one attribute or reaction of the malignant cell that is readily demonstrable or measurable has yet been shown to be the exclusive and specific property of cancer tissue. Obviously there are many ways of defining the malignant condition, and such definitions as a growth of tissue that mimics the histological structure of a normal organ or structure, and yet kills by metastases and death, while acceptable for many purposes, rests upon a combination of clinical and pathological findings, rather than upon a demonstrable and measurable laboratory finding. The blind trial and error method of applying one dye after another to tumor cells, in the hope of a chance unique combination, as tried by some observers, is an admission of this lack of any reliable data on the specific property or properties of the malignant cell. And yet, on critical analysis, a study of various reactions involved in malignancy appears a far more fruitful approach to the problem of differential staining than the trial and error methods of routinely applying successive dyes. The paramount reason for such a roundabout approach to the problem is the delicacy of the reactions involved. Unless we have in mind the order of changes which the dye is supposed to indicate, our earliest successes in this direction, definite but not pronounced, are in danger of being entirely overlooked and further slight modifications to emphasize such findings will not be undertaken. In fact all of the various routine stains now in use in the laboratory, by identifying the histological structure of the cancer, with that of the normal tissue of the organ of origin, rather than differentiating it therefrom, seem to indicate that some more refined order of staining is essential to the problem in hand. Thus, as is plainly evident from a review of many of the recent findings in regard to malignancy, the difference between the cancer and the normal cell is more of degree than of kind, and the dye employed to demonstrate such differences more probably will act as "an indicator dye," than a direct "combining reagent." Therefore, if we lay aside the hope for a *chance specific combination* between a dye and cancer tissue, a search into the more subtle characteristics of malignancy is in order. The demonstration

of such characteristics by staining methods would be just as useful, if specific, as the more outspoken reactions.

In the investigation of these less obvious characteristics of cancer tissue three major fields present themselves. The *first* involves the quest for some structural feature within the malignant cell which may be proved specific for cancer. The employment of dyes in such a cytological undertaking is for the purpose of portraying some intimate peculiarity of cell structure heretofore overlooked by the cruder routine methods of pathology.

A *second* line of investigation rests upon a new conception in the pathology of tumors. It makes use of the biological fact that the tumor cells are not merely growing by multiplication but are passing thru definite cycles of progressive histogenic differentiation. The use of dyes in defining such histogenic cycles of malignant growth is for bringing out more clearly the extent and order of the successive stages thus exhibited by cancer tissue, in the hope of demonstrating their specificity for malignancy.

The *third* realm of inquiry applies some of the newer biochemical methods to tumors. It makes use of the fact that while the metabolic activities of cancer tissue such as glycolysis and oxidation, do not differ in kind from those of other tissues, they are proceeding at a *rate of higher intensity* and that certain indicator dyes (such as those recently studied by Mansfield Clark) are adaptable to the measurement of the intensity of such reactions.

A. CYTOLOGY OF THE CANCER CELL

The work of Lipschütz has been fundamental in calling attention to the finer structural characteristics of the cancer cell. While such workers as MacCarty of the Mayo Clinic and Boveri have endeavored to set up some peculiarity of the cell nucleus as specific for cancer, the prominence of the nucleus and its nucleolus, emphasized by MacCarty and the unique mitosis stressed by Boveri,[13] are by no means constant findings in malignant tissues nor limited to these tissues.

In the work of Lipschütz, a finely granular body just outside the nucleus of the cell, varying in its morphology from a paranuclear mass next the nucleus to a perinuclear ring surrounding the achroplasm of the cell, is stressed as a specific cytological feature of malignant tissue. This cellular structure, termed by its discoverer the "plastin reaction" varies in its staining reaction, sometimes being achromatic, sometimes strongly basophilic. The demonstration

[13]Boveri, T. The origin of Malignant Tumors. Williams & Wilkins, Baltimore. 1929.

of this "plastin reaction" in the hands of Lipschütz depends upon an extremely carefully fixed tissue technic, and is at best extraordinarily difficult of perception. So far, the only stains successful in bringing out this unique cellular structure have been the Giemsa stain and Heidenhain's Hematoxylin.

From the standpoint of differential staining for tissue diagnosis the findings of Lipschütz constitute a valuable lead. At present his "plastin reaction" has neither been proved specific nor constant for all forms of malignancy, and the reaction is much too delicate and too difficult of demonstration to be of practical diagnostic value.

Experimentation carried out along these lines offers the advantages of a definite cancer characteristic to which we are attempting to apply the chemical reaction of dyes, rather than a blind trial and error method. In addition a definite biochemical lead in the selection of dyes is afforded by the knowledge that the Giemsa and Heidenhain dyes are capable of demonstrating this structure, while the other various routine laboratory dyes are not.

B. THE HISTOGENIC CYCLE OF CANCER IN RELATION TO DIFFERENTIAL STAINING

The demonstration of definite histogenic cycles for the various forms of malignancy is a newer realm of investigation than of cellular morphology in cancer and implies a more extensive group of staining technics. The underlying principle in this field of investigation is the finding that all malignant tissues are not only undergoing rapid cell multiplication, but are also going rapidly thru a series of stages or cycles of tissue differentiation. As shown by Geschickter for osteogenic sarcoma the general character of this histogenic cycle is not peculiar to malignancy, but duplicates in its essentials the normal embryonic cycle of the tissue from which the tumor arises. Thus chondrosarcoma duplicates in its various stages of cell differentiation the embryonic differentiation of cartilage, and squamous cell carcinoma similarly duplicates the embryology of epidermal tissue. This identity of histogenic cycle common to both the embryo and malignancy is rather an advantage than a hindrance to differential microscopic diagnosis, because clinically we are dealing with post-natal and not pre-natal tissues.

But in attempting to use the histogenic cycle of malignancy as a diagnostic criterion, three other sources of confusion arise. For this embryonic cycle of tissue differentiation is found also in (1) normal growth zones of actively growing organs (2) embryonic rests and (3) certain benign tumors. Thus the histogenic cycle characteristic

of chondrosarcoma (a tissue differentiation from mesemchymal connective tissue—to cartilage and to bone) is not only typical of the embryo, but is also found in normal growth zones about the knee joint, in embryonic rests such as multiple exostoses, and in benign tumors such as chondromatosis of the joint cavities. It is just at this point in differentiating between such benign and malignant growth cycles that the value and applicability of differential staining to this feature of malignancy comes to the fore.

A constant alteration of the chemical composition of the cellular structures along with their morphology is a common feature of all forms of tissue differentiation. But in all benign growths the end stage of the cellular differentiation is fully reached, and the chemical composition of the cell products such as the osseous matrix of bone or the keratin of epithelial tissues reaches a final stable form. On the other hand, in malignancy the end stage of differentiation is imperfectly and only partially achieved so that abortive and premature cellular products predominate. In this way a dye which reacts specifically with the end product of tissue differentiation will fail to show its dominantly characteristic color change in malignant conditions. Thus in osteogenic sarcoma the manufacture of a true osseous substance is never fully achieved and a stain specific for this substance will not give its typical reaction in the presence of this form of malignancy.

An elaboration of this method of differential staining necessitates the extension of our present limited group of stains specific for the various basic tissues. It also leads inevitably to the use of polychrome staining reagents or more specifically to an admixture of dyes, or a group of staining reagents, instead of one specific dye for malignancy. For obviously a stain which is specifically adapted to an end product of one type of tissue is not applicable to another, and a different dye is necessary when a tumor from a different organ or tissue is tested. Fortunately there is a limit upon the number of different end products to be tested because the great number of clinical and pathological cancer entities fall readily into a few large histogenic classes, and these basic histogenic classes can be accurately defined and catalogued by the proper embryological studies.

C. THE USE OF DYES IN MEASURING THE METABOLISM INTENSITY OF MALIGNANT TISSUES

The third domain for investigation in the quest for a cancer characteristic suitable for differential staining deals with the intensity and rate of the biochemical reactions of malignant tissues.

The work of Warburg and his associates on the glycolysis and lactic acid production in tumor tissues has been outstanding in initiating experimental work on the biochemistry of cancer. Unfortunately, neither the amount of lactic acid produced, nor the rate of anaerobic glycolysis has proved definitely specific for malignant tumors, and similar researches by Bierich aimed at the disclosure of some other metabolic products of cancer such as the elaboration of organic sulfur compounds, have not yielded specific findings.

Among the experimental findings of Warburg, however, the demonstration that cancer tissue is able to survive in an absence of oxygen if glucose is provided in physiological solution, while other tissues succumb, offers definite possibilities for differential staining. For, if normal and cancer tissue are placed in physiological solution containing glucose, and oxygen is withheld by a constant streaming atmosphere of nitrogen, the normal tissue should be first to show signs of degenerative changes. Availing ourselves now of the experimental information that healthy cells are impervious to dyes because of the intact cell membrane, while degenerative cells in the earliest portion of their moribund condition tend to absorb such stains, it is possible to use such a test in differentiating cancer from other tissues. With such a method the cells of the malignant tissue would fail to stain; in contrast to the non-malignant, which would absorb the dye.

There are certain inherent, but not insurmountable difficulties in such a method, which is now being tried experimentally in this laboratory. Foremost among these is the time required for the degenerative changes to make their appearances in the non-malignant tissue. The time, however, may be minimized in several ways, first by increasing the concentration of the dye to a point approaching toxicity; second by employing vital dyes which tend to gain entrance to the non-cancerous cells more readily than the non-vital dyes (while cancer tissue is notably refractory to vital staining) and third by a preliminary washing of the tissue with normal saline to remove any nutritive elements remaining in the tissue fluids at the time of the operative removal of the specimen.

Another difficulty to be surmounted is the invariable presence in cancer tissue of degenerative changes. This may be counteracted in part by choosing the tissue from a sound portion of the growth, avoiding areas of central necrosis, hemorrhage, secondary infection, etc.

III. The Choice of Dyes for the Differential Staining of Cancer

Books devoted to stain technology and the catalogs of commercial concerns devoted to the manufacture and sale of such biological stains, disclose the fact that there are now in use over 1000 stain solutions in biological experimentation. New dyes, remaining to be applied to biology, are increasing by the hundreds as a result of chemical investigations, and the possible modifications of the solution in which the dyes are employed raises the number of dye reagents toward infinity. In endeavoring to select from such an indefinite number of permutations and combinations the proper dye reagent for the differential diagnosis of cancer, we are hopelessly floundering unless our choice is intelligently restricted to definite groups of reagents selected on the basis of a well defined conception of the requirements of the problem in hand.

In the foregoing discussion there has been set up a definite group of such requirements, and with these requirements in mind, it now remains to indicate the groups of staining reagents most likely to be of service in the problem of the differential staining of cancer.

A. A specific stain for the cytology of cancer

Lipschütz has found the azur-eosin mixture of Giemsa prepared by G. Grübler and Company of Leipzig of special value in staining the morphological peculiarity of cancer designated by him the plastin reaction—or "nuclear hood." This "nuclear hood" may also be demonstrated by its failure to stain with Heidenhain's hematoxylin solution. He has also found that only three or four of a wide variety of fixing fluids may be used in conjunction with these stains. These fixtures are Zenker's fluid minus acetic acid, Helley's fluid, Regaud's fluid, and the sublimated alcohol of Schaudin.

In endeavoring to modify these staining technics of fixed tissues to portray this unique structure of the cancer cell more definitely, two outstanding principles are at once evident. *First* the staining reagents are not new or unique but of the common routine variety: methylene blue, eosin, hematoxylin. The fact that Giemsa's particular modification of the azur-eosin solution of Romanowsky should bring out the plastin reaction, while innumerable similar modifications of the same reagents by Wright, Wilson, Reuter, Leishman, Jenner, May-Grunwald, etc., should fail to do so, points to the fact that it is the chemical and physical properties of the prepared solution rather

than the dye reagent used that is the determining factor.[14] This fact is borne out by the similar character of Heidenhain's hematoxylin, a modification only, of a routine stain.

Second, a valuable clue on the fundamental characteristics of the prepared solution involved in this specific staining reaction is made available by an analysis of the fixing fluids which are essential to the proper functioning of the dye. Definitely alkaline, acid, oxidizing or reducing, fixing solutions destroy the specific staining reactions of the dye.

The problem then in specifically staining the morphological peculiarities of cancer tissue may be, therefore, clearly defined. It depends on controlling the hydrogen-ion concentration, and the oxidation-reduction intensity of both the fixing fluids and the staining reagents employed, using as our staining reagents a "dye-system" of the azur-eosin series, or of the hematoxylin category with some suitable counterstain.

B. SPECIFIC STAINS FOR THE END PRODUCTS OF HISTOGENICAL CYCLES

It is too early at this date to list the various basic histogenic tissues and give specific stains for their fully differentiated forms. Mallory's

[14]In working out modifications of the Giemsa staining technic referred to in this paper, changes in the dye solutions were made in three different respects. First, it was found possible to use dye mixtures in which the compounds were present in more simple and more highly pure form. The Giemsa stain can be modified for fixed tissue by employing eosinated substitution products of the various derivatives of thionin and using these in mixture with smaller amounts of pure azur A (also a thionin derivative). Basic fuchsin is added in amounts equal to the azur A, and aids greatly in the differential qualities and intensity of the staining reaction. Of thirteen eosinates of various thionin derivatives used in mixture with azur A and basic fuchsin, the eosinate of victoria blue B was found to be one of the best. The dyes are used in alcoholic solution and the staining reaction is obtained by covering the slide with the deparaffinated section with the dye for thirty seconds. Sections are then washed with water and cleared in acetone-xylol mixtures.

The other two changes employed were made for the purpose of adapting the above staining reaction to unfixed frozen sections. Since alcoholic solutions cannot be used and the eosinates are insoluble in water, ethylene glycol with 20% absolute alcohol was used for dissolving the eosinates and the azur A and fuchsin were added in aqueous solution. For unfixed frozen tissue, acid fuchsin was substituted for the basic fuchsin; and the fuchsin and azur were used in amounts equal to the eosinates. Thus by substituting ethylene glycol for alcohol and by changing from a basic dye to an acid dye, a staining reaction similar to the Giemsa stain for fixed tissue was obtained for frozen sections. The acid fuchsin makes the dye mixture basic in reaction which is an essential in staining unfixed tissues which we know are definitely acid. Sections are satisfactorily stained by immersion in the dye mixture for from fifteen to thirty seconds.

These results will be reported in greater detail as soon as the best possible mixtures of these various eosinate combinations are determined by more extensive studies.

connective tissue stain, mucicarmin for cartilage, and a modified silver impregnation technic for osseous tissue are among the specific stains proposed for various connective tissue products. Similar stains have been advocated for epithelial and neural structures. Methyl green has proved of value in this laboratory for the demonstration of lymphatic tissues.

This roundabout method of arriving at specific stains applicable to the differential diagnosis of cancer is not proposed solely for its diagnostic value in malignancy. Its fruitfulness as a field of investigation rests upon its usefulness in tracing the histogenesis of various forms of malignancy and throwing valuable light on the mode of origin of various tumors.

C. SPECIFIC STAINS FOR THE BIOCHEMICAL PROPERTIES OF CANCER

In a discussion of the Warburg findings in regard to the metabolism of malignancy, the possible application of the viability of cancer under anaerobic conditions, to the differential diagnosis of cancer by vital staining was pointed out. The early degeneration of non-malignant tissues under anaerobic conditions should render them permeable to dyes in contrast to the more viable malignant tissues which should remain refractory to staining. It was suggested that one of the vital stains ordinarily not absorbed by either malignant or normal tissues (except those cells of the reticulo endothelial apparatus) should be tried. Congo red is admirably suited to this purpose as experiments with this dye have shown that degenerating cells are readily permeable by it, while ordinarily normal and malignant tissues are not. Both the concentration of the dye and its colloidal state are important factors in determining the rate of its absorption by degenerating cells, and these two features may be made the subject for a carefully graded series of variations. Other dyes such as trypan blue, which are not absorbed by viable tumor cells, are being tried.

Conclusions

In addition to the three fundamental fields of microscopic technic, analysis of specific cancer properties, and selection of suitable dyes—a final and fourth fundamental requirement for the problem of differential staining in cancer has been reserved for discussion here. Granted that by the use of the proper microscopic method, and using a characteristic property of cancer tissue, we have been able to select a suitable dye to indicate this property, there still remains the problem of proving the specificity of the reaction by demonstrat-

ing its constancy for all types of human malignancy and its failure to appear in all other diseased conditions and normal tissues.

In contemplating this final and exhaustive test for a differential staining method in cancer, it becomes evident that a comprehensive reservoir of human pathological material is essential, and this material must be available for experimentation in the particular microscopic form of preparation suitable to the methods of differential staining under development. It should also be apparent how costly and wasteful of money, time and energy such an exhaustive test of a differential staining method would be, if the staining method developed should prove hopelessly inadequate, because not developed on a basis of representative material. It may therefore not be amiss to point out two fundamental shortcomings which are prevalent everywhere in cancer experimentation. These two shortcomings which have a direct bearing on the dye problems in cancer are the willingness everywhere to employ animal tumors, on the assumption that they are identical in all properties with human tumors, and second the assumption that all forms of malignancy in whatever tissue or organ they arise are necessarily identical in all of their characteristics. I believe it is well to doubt the validity of both of these assumptions, because beyond question they are assumptions and not yet proved.[15] Behind the current, ubiquitous, willingness to accept these two assumptions is the desire on the part of cancer experimenters to leap to the discovery of the cause or cure for cancer, rather than to patiently develop a scientific body of information on the properties and fundamental characteristics of cancer as a purely biological phenomenon.

A more open-minded consideration of the present status of information in regard to cancer should warn us, that being in all probability a local tissue disease, arising in the tissues themselves (and not an outside "weed" such as an infection) the characteristics of malignancy probably vary in some degree according to which one of the several basic tissues the disease takes as its source. Therefore, in experimenting with the problem of differential staining in cancer it would seem wise to employ human tissues, and to choose a variety of the basic tissues which may be involved by cancer.

[15]Regaud has conclusively proved that the different forms of cancer vary in regard to their radio sensitivity according to their tissues of origin.

ABSORPTION RATIOS OF BIOLOGICAL STAINS

By W. C. Holmes[1] and A. R. Peterson[2]

(Contribution No. 174 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.)

Abstract—**Absorption ratios are supplied for most of the dyes used as biological stains.**

The use of these ratios will enable the analyst to identify the stains conveniently, and will also frequently afford information of considerable value respecting their purity.

The term absorption ratio, herein employed, may be defined as the ratio of the extinction coefficients of the solution of a colored substance at two specific wave lengths. If the wave lengths employed are selected on opposite sides of the absorption maximum of the coloring matter, the absorption ratio defines the spectral position of the absorption band.[3] If they are selected on the same side of the maximum, it defines the gradient of the slope of the band within the region of measurements. In either instance the absorption ratio affords a valuable means of differentiating between different dyes, since both the spectral locations of absorption bands and the gradients of their slopes in specific regions are highly characteristic in general, for individual dyes.

It is evident, furthermore, that such ratios are well adapted to reveal the presence of subsidiary coloring matters in dyes and should generally afford a valuable criterion of the relative purity of a dye sample in that respect.

The absorption ratios of about 500 dye samples were measured, including samples of the great majority of dyes which find extensive application in biological staining. The objectives in view were those of obtaining evidence respecting the degree of chemical variation in market supplies of dyes and stains, of establishing standards by which such products may be judged; and of determining in what degree the employment of absorption ratios may be relied upon as a practical means for revealing the relative purity as well as the identity of individual stains.

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[3]W. C. Holmes. Spectrophotometric identification of dyes. III. Basic violets of the triphenyl-methane group. Ind. & Eng. Chem. **17**, 918. 1925.

The standard solvent employed was a mixture of equal volumes of water and 95% alcohol.[4] The principal advantages of this solvent over water are those of inhibiting the deposition of basic dyes on the glass surface of the absorption cells and of preventing the tautomeric alterations which many dyes undergo with changes in dye concentration in aqueous solutions.[5] With this solvent any regulation of dye concentration is unnecessary. In general, sufficient dye was dissolved in about 50 cc. of solvent to afford convenient readings in a half-centimeter layer. In some instances it appeared advisable to render such solutions moderately acid or alkaline, and with a few dyes it was necessary to employ a different solvent.

The measurements were carried out with a direct reading Bausch and Lomb spectrophotometer of the most recent type, equipped with cup and plunger absorption cells in which the solution layers could be regulated rapidly and accurately to insure optimum conditions for all readings. With this convenient instrument it was found possible to determine absorption ratios very rapidly. The entire operation of preparing the solutions and making the photometric measurements need require only a few minutes.

The accuracy with which absorption ratios may be determined varies with the photometric range employed, which may be regulated to conform with optimum conditions for the individual operator, and with the spectral range in which the measurements must be made. Visual sensitivity is greatest near the middle of the visual spectrum and decreases as either end is approached. At the extremes of the working range employed, the authors were usually able to check results within one or two per cent. Elsewhere practically identical ratios were frequently obtained, and the probable error in the determination of the majority of ratios is small.

Absorption ratios for dyes determined in this manner are recorded in Table 1. The solvent denoted therein as "Reg." is the standard solvent previously described. In each instance the absorption ratio (R) is calculated by dividing the extinction obtained at the lower wave length specified by that obtained at the higher wave length.

Of the unindexed dyes tabulated, magenta II is the chloride of triamino phenyl ditolyl carbinol anhydride, synthesized by J. T. Scanlan of this Division. The cresyl echt violet (Grübler) is of unknown constitution. The samples examined are representative

[4]W. C. Holmes. The choice of solvent in spectrophotometric dye analysis. Am. Dyestuff Reporter, April, 1926.

[5]W. C. Holmes. The influence of variation in concentration on the absorption spectra of dye solutions. Ind. & Eng. Chem. **16**, 35. 1924.

TABLE 1. ABSORPTION RATIOS OF STAINS

Dye	C. I. No.	Solvent	Wave Lengths: (mμ)	R
Fluorescein, Na salt	766	Reg. + 5 drops 1% Na_2CO_3	500, 530	36.5
Auramin	655	Reg.	" "	30.0
Martius yellow	9	"	" "	14.8
Orange G	27	"	" "	5.24
Resorcin yellow	148	"	" "	3.61
Chrysoidin Y	20	"	" "	2.83
Chrysoidin R	21	"	" "	2.63
Sudan I	24	95% alcohol	" "	2.49
Diamond flavine	110	Reg.	" "	2.45
Orange II	151	"	" "	1.95
Bismark brown Y	321	"	" "	1.88
Orange IV	143	" + 10 drops 6N. HCl	" "	1.75
Bismark brown R	35	Reg.	" "	1.58
Sudan II	73	95% alcohol	" "	1.26
Congo red	370	Reg.	" "	1.23
Purpurin 4 B	448	"	" "	1.19
Vital red	456	"	" "	1.17
Sudan III	248	95% alcohol	" "	1.11
Crystal ponceau	89	Reg.	" "	1.04
Biebrich scarlet	280	"	" "	1.00
Orange I	150	1% Na_2CO_3	" "	1.00
Sudan IV	258	95% alcohol	" "	0.97
Fast red A	176	Reg.	" "	0.97
Methyl orange	142	" + 10 drops 6N. HCl	" "	0.97
Bordeaux B	88	Reg.	" "	0.88
Dibromofluorescein	—	" + 5 drops 1% Na_2CO_3	505, 535	3.84
Mercurochrome	—	" " " " " "	" "	2.83
Eosin	768	" " " " " "	" "	0.92
Janus red	266	Reg.	510, 540	1.12
Amaranth	184	"	" "	1.07
Eosin B	771	" + 5 drops 1% Na_2CO_3	515, 545	1.41
Phenosafranin	840	Reg.	" "	1.13
Erythrosin	773	" + 5 drops 1% Na_2CO_3	" "	1.04
Safranin	841	Reg.	" "	1.02
Phloxine B	778	" + 5 drops 1% Na_2CO_3	" "	0.97
Methyl eosin	769	" " " " " "	" "	0.96
Ethyl eosin	770	" " " " " "	" "	0.86
Neutral red	825	" + 5 drops glac. acetic acid	" "	0.75
Methylene violet 2 R	842	Reg.	" "	0.61
Rhodamin S	743	"	530, 560	1.51
Para rosanilin	676	"	" "	1.13
Neutral violet	826	" + 5 drops glac. acetic acid	" "	1.05
Magenta II	—	Reg.	" "	0.96
Rosanilin	—	"	" "	0.89
Acid fuchsin	692	"	" "	0.81

TABLE 1. ABSORPTION RATIOS OF STAINS—Cont.

Dye	C. I. No.	Solvent	Wave Lengths: (mμ)	R
Rhodamin G	750	Reg.	530, 560	0.78
Pyronin G	739	"	" "	0.775
New fuchsin	678	"	" "	0.755
Rhodamin B	749	"	" "	0.58
Pyronin B	741	"	" "	0.52
Rose bengal 3 B	779	" +5 drops 1% Na_2CO_3	540, 570	0.92
Alizarin red S	1034	N. NaOH	545, 575	1.21
Alizarin	1027	" "	" "	0.81
Chrome black F	299	Reg.	" "	0.94
Magdala red	857	"	" "	0.90
Meldola's blue	909	"	560, 590	1.13
Methyl violet	680	"	575, 605	1.405
Iris violet	847	"	" "	1.29
Benzyl violet	683	"	" "	1.14
Anilin blue (s. sol)	689	"	" "	1.08
Crystal violet	681	"	" "	1.065
Nigrosin (w. sol.)	865	"	" "	1.01
Ethyl violet	682	"	" "	0.91
Thionin	920	"	590, 620	1.44
Cresyl echt violet (Gr.)	—	"	" "	1.20
Anilin blue (w. sol.)	707	"	" "	1.08
Trypan blue	477	"	" "	1.08
Niagara sky blue	520	"	" "	0.99
Methyl blue	706	"	" "	0.97
Methylene violet (Bernthsen)	—	"	" "	0.835
Indigotin	1180	"	" "	0.79
Victoria blue B	729	"	605, 635	1.18
Night blue	731	"	" "	1.04
Brilliant cresyl blue	877	"	" "	0.90
Malachite green	657	"	" "	0.83
Fast green FCF	—	"	" "	0.76
Guinea green B	666	"	" "	0.715
Brilliant green	662	"	620, 650	1.51
New methylene blue N	927	"	" "	1.21
Cresyl violet (NAC)	—	"	" "	1.16
Toluidine blue O	925	"	" "	1.04
Light green SFY	670	"	" "	0.98
Nile blue A	913	"	" "	0.90
Methyl green	684	"	" "	0.90
Methylene green	924	"	635, 665	1.12
Janus green	133	"	" "	0.97
Nile blue BB	914	"	" "	0.93
Methylene blue	922	"	" "	0.59
Thionin blue	926	"	" "	0.51
Capri blue GON	876	"	650, 680	1.27

of the product which the Grübler Company now supplies for biological staining, and appear to be identical with the dye marketed by Leonhardt. The American stain cresyl violet (NAC) is aminonaphtho-dimethylamino-tolazoxonium chloride. Methylene violet (Bernthsen) is dimethyl thionolin. Fast green FCF is the p-hydroxy analog of erioglaucine A (C. I. 671).

Of the comparatively few stains omitted from the table no samples were available in some instances. In others as, for example, the less completely substituted types of erythrosin, phloxine, and rose bengal, the available samples were of such a divergent character that no reliable conclusion could be reached respecting their normal characteristics. Only two dyes were encountered, picric acid and narcein, which proved unsuited for examination within the spectral range in which it was considered practical to undertake visual measurements.

Some of the recorded ratios doubtless differ, in a minor degree from correct ratios for the pure dyes. The value given for methylene blue, for example, is slightly higher than the corresponding value for pure methylene blue. The recorded values are intended to be representative of market products and, in general, of the purest grades of such products. They will be used as provisional working standards for the future examinations of the stains in question. In general they may be accepted as reliable, altho it may eventually prove advisable to revise in a minor degree the values given for eosin B, Nile blue A, methylene green, and a few other dyes of which the available samples were of a somewhat diverse character.

A comparison of results obtained with different samples of individual dyes indicates essential uniformity in market supplies of most stains, but considerable diversity in some instances. Values obtained with acid fuchsins, for example, ranged between .75 and .95. The recorded value for this dye (.81) was obtained with a sulfonated rosanilin, prepared by J. T. Scanlan of this Division, which has proved exceptionally satisfactory in practical staining application. In other instances of dyes known to be mixtures, such as ordinary basic fuchsin, safranin, and methyl violet, the products of different manufacturers proved surprisingly uniform. Examples of dyes of supposedly homogeneous type which proved variable were found in most of the dye groups, but more frequently, perhaps, in the phthalein and oxazin groups than elsewhere.

In view of such variations it is obvious that the identification of stains by means of their absorption ratios necessitates the exercise of discretion. Abnormal samples may frequently be encountered

which give somewhat different values from those recorded. It will probably be but seldom, however, that such samples will contain sufficient proportions of subsidiary coloring matters to obscure seriously the identity of their principal component.

In general the ratios are less reliable for most yellow, orange, and brown stains than for those of other colors, because the gradient of the slope of an absorption band is less characteristic than its spectral location. They are less characteristic for azo dyes than for the dyes of other classes because their bands are broader and less well-defined.

With these qualifications, and with the proviso that it may sometimes prove advisable to resort to other methods for corroboratory evidence, the absorption ratio may be advocated as an extremely convenient and valuable means for identifying stains. The recorded values for chrysoidin Y and R, pararosanilin, and rosanilin, crystal and benzyl violets, and methylene and thionin blues, for example, illustrate how decisively they may enable the analyst to distinguish between very similar dyes which may be differentiated by other means only with extreme difficulty or considerable labor.

In connection with this application of absorption ratios it was considered advisable to investigate with considerable care the possible influence of the salt-forming radical associated with the dye. It has been shown[6] that the color and absorption of various metallic salts of polyhydroxy anthraquinone dyes are decidedly different, and the senior author has observed analogous variations with metallic salts of direct azo dyes. Some biological stains are marketed in different forms; basic dyes, in particular, appearing as salts of different acids. In order to determine whether the absorption ratios might differ in such instances, a considerable variety of different salts of the same dyes were compared. The most comprehensive tests were carried out with rosanilin, of which the nitrate, sulfate, acetate, iodide, borate, citrate, bromide, benzoate, oxalate, chloride and resorcin addition products were prepared and examined. All of the ratios with this series ranged between .88 and .90. The conclusion was reached that the influence of the salt-forming radical is entirely negligible in any form of product in which stains are likely to be marketed.

The value of absorption ratios as a criterion of dye purity was also demonstrated conclusively. In the technic followed in obtaining

[6]D. B. Meek and E. R. Watson. The colour of polyhydroxyanthraquinone dyes. J. Chem Soc. (London) **109** (2), 544. 1916.

the recorded values, this aspect of the matter was given less consideration than that of dye identification, and it is probable that the conditions employed in some instances were not equally suitable for both purposes. This was shown to be true with indigotin. The fact that a sample of the dye which was known to be somewhat abnormal proved indistinguishable from normal samples when examined in the standard solvent led to further investigation. It was found that the mono-, di-, tri- and tetrasulfonates of indigo gave ratios, respectively, of .76, .78, .90, and 1.39 under those conditions. It is obvious, accordingly, that the presence of the lower sulfonate in indigotin would escape detection in the solvent specified, altho the presence of appreciable proportions of higher sulfonates would be apparent. Since the lower sulfonate is the principal impurity found in commercial samples of indigotin, the determination of the absorption ratio under these conditions affords no adequate criterion of dye purity. It was found, however, that the ratio values of the di- and monosulfonates were, respectively, .84 and 1.36 when the same wave lengths were employed and the dyes dissolved in 80% alcohol, and it is evident that the presence of relatively small proportions of the lower sulfonate in indigotin will be apparent if the dye is examined in the latter solvent.

Altho it is probable that the wave lengths, solvents and other conditions employed in obtaining the recorded absorption ratios are not always exceptionally well-adapted for the determination of dye purity, it is evident that they are in general relatively adequate for that purpose. It has been shown[7] that a ratio of the same type may be used in determining the purity of methylene blue within relatively narrow limits, and the decided variations in recorded values for related dyes indicate that such values may frequently afford a roughly quantitative, rather than merely qualitative, measure of subsidiary dyes which may be present in impure dye samples. Dibromo fluorescein, for example, gives a value of 3.82 under the same conditions in which ordinary eosin gives a ratio of .92, and the ratio plainly affords a very satisfactory means of determining the degree of under-bromination which may be found in eosins. Even greater differences between dyes and the subsidiary coloring matters with which they are associated are not uncommon. Crystal violet gives a value of approximately 11.3 when examined under the conditions employed in testing methyl green, which gives a

[7] W. C. Holmes. The spectrophotometric evaluation of mixtures of methylene blue and trimethyl thionin. Stain Techn. 3, 45, 1928.

value of .90. The oxazones which appear to be invariably present in oxazin dyes differ in color in a very considerable degree from the dyes which they contaminate.

Absorption ratios, accordingly, supply a remarkably convenient means for establishing the identity of stains, in general, and for determining the degree of purity of many stains in as definite a degree as is ordinarily necessary or desirable.

FURTHER EXPERIMENTATION WITH A NEW DIFFERENTIAL STAINING METHOD FOR CONNECTIVE TISSUE COMBINED WITH THE ORDINARY HEMATOXYLIN-EOSIN STAIN

EDITH E. LARSON, *Department of Zoology, University of Kansas*

There appeared recently in the University of Oklahoma Bulletin an article by Jos. M. Thuringer[1] describing a new differential staining method for connective tissue. Dr. W. J. Baumgartner suggested to the writer that she undertake to stain a series of sections using the method described by Mr. Thuringer. We wished especially to test this method in order to determine if it could be introduced into the course in technic as one of the routine stains for connective tissue.

PROCEDURE

According to my observation, tissues fixed in any one of the more common fixing fluids stained equally well when subjected to this method. Paraffin or celloidin sections may be used.

The sections were stained in hematein solution[2] two to three minutes. I found that the more deeply stained preparations were better. The contrast with the following counterstains was, then, more outstanding. No destaining was done as suggested by Thuringer. After the tissues had been treated with tap water they were stained with eosin B (0.5 gram eosin B, Coleman & Bell, to 100 c.c. of 25% alcohol) for one minute or slightly longer. The tissues then were passed thru the series of alcohols to 95% and were treated with orange G (0.5 gram orange G, Coleman & Bell, to 100 c.c. 95% alcohol) for fifteen seconds or less.[3] Then they were dehydrated with absolute alcohol and cleared and mounted.

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[1]Thuringer, Jos. M., A new differential staining method for connective tissue combined with the ordinary hematoxylin-eosin stain. University of Oklahoma Bulletin Vol. 2. October, 1922. New series No. 247.

[2]This solution was made up similarly to Delafield's hematoxylin except that hematein (Coleman and Bell) was used, and ripening was omitted. One gram hematein was dissolved in 10 cc. absolute alcohol and added drop to drop to 100 cc. saturated aqueous ammonia alum. After filtering 25 cc. glycerin and 25 cc. methyl alcohol was added.

[3]These were not Commission certified dyes, and accordingly the exact dye content is not known.

Discussion

The best demonstrations of the method were obtained on skin and stomach sections. In skin sections one can easily recognize the connective tissue fibers because they are stained a distinct yellow against the pink background. Not only are the bundles of white inelastic connective tissue fibers stained vividly by the orange G, but also the individual threads of the yellow elastic fibers. The contrast of blue epithelium, pink cytoplasm, and yellow connective tissue fibers is very striking. In the stomach sections the connective tissue of the submucosa is distinctly marked off because of its yellow color. The connective tissue fibers between the muscle fibers are also easily seen. In such an organ as the testes where there is so little connective tissue between the tubules, this method is even more satisfactory because it brings out the little connective tissue there is in contrast to the other tissue elements.

Conclusion

I am convinced, as a result of these experiments, that this is a good staining method and feel that it deserves to become a common one for ordinary laboratory staining. Its chief virtues are; (1) the contrast which it gives the connective tissue with reference to the other tissue elements, and (2) the simplicity of the technic.

My thanks are due Dr. Baumgartner for many helpful suggestions.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

AOYAMA, FUMIO. Eine Modifikation der Cajakschen Methode zur Darstellung des Golgischen Binnenapparates. Zts. Wis. Mikr., **46**, 489–491. 1930.

The cadmium formol method presents clearly the Gologi apparatus in various cells and succeeds in relatively large pieces.

1. Fix small pieces of tissue in the following fluid 3 to 4 hours:

Cadmium chloride	1.
Formol, neutral	15.
Dist. water	85

2. Rinse quickly in two changes of distilled water and transfer to 1.5% solution of silver nitrate for 10 to 15 hours at 22° C.
3. Rinse quickly in two changes of distilled water preferably in a dark room and transfer for 5 to 10 hours to the following reducing solution:

Hydrochinon	1
Formol, neutral	15
Dist. water	85
Sodium sulfite	0.1 to 0.15 (a quantity which will produce a yellowish tinge)

4. Wash thoroly in tap water, run up thru the alcohols, embed and cut 3 to 4 μ thick.
5. Mount and stain as desired with carmin or hematoxylin-eosin.

The length of silver impregnation depends upon the temperature and may be regulated accordingly. Epithelial cells of the alimentary tract or of the uro-genital system require only 1 to 2 hours fixation. Silver nitrate in 0.5% solution also gives satisfactory results if allowed to act for 36 to 48 hours. Various organs of amphibians, reptiles, birds and mammals may be thus prepared. Cold blooded animals seem to require longer fixation and impregnation than do warm blooded ones.

BUBENAITE, J. Über einige Erfahrungen mit der Golgi-Methode. Zts. Wis. Mikr. **46**, 359–360. 1929.

The author proposes a fast procedure for the Golgi silver impregnation:

1. Fix 1 to 2 days in 10% formalin;
2. Transfer to Müller's solution or 2.5% aqu. potassium bichromate for 2 days at 34°C.
3. Rinse in 2% $AgNO_3$ and transfer to same for 1 or 2 days at 34°C.

Embed and mount as usual.

Altho one is not certain which elements will show up better, this is a quick, perfectly dependable method for class preparations. Landau's synarmotic cells of the cerebellum, generally hard to treat, are frequently impregnated as well as the other ganglionic cells.

DAVIES, J. I. A method of orienting difficult embryological material for sectioning. Anat. Rec. **43**, 381–385. 1929.

For the early stages of the frog proceed as follows: Cut a rectangular block of tissue about 8x4x4 mm., preferably brain kept some time in alcohol, dry with a towel, and with a scalpel dig out a cavity a little larger than the egg, but not deeper than its diameter. Put a drop of Meyer's albumen into the cavity and transfer the unfixed egg (previously rolled to remove the jelly), with hardly any fluid in the cavity. Rotate egg under binocular so that yolk plug is uppermost. Gently drop fixing fluid; this will coagulate the albumen and the egg will stay in the desired position. Transfer to fixing fluid to complete fixation.

Dehydrate and mount, maintaining the rectangular shape of the tissue. Mount on microtome block so that it conforms to the shape of the tissue. According to whether one cuts parallel to the largest or to the longest surface of the block of tissue, the sections will be transverse or longitudinal, either horizontal or sagittal. One may judge whether the sections pass from the anterior to the posterior region or vice versa. The relation between the dorsal, ventral or lateral lips may be studied.

The method is also applicable to various fixed materials, and in that case the objects are transferred from alcohol and fixed with alcohol of the same strength as that in which they have been kept. The advantage in using fixed material is that it can be stained in bulk before embedding in the tissue. The brain is then almost unstained (counterstained only), the effect being similar to that obtained with celloidin embedding.

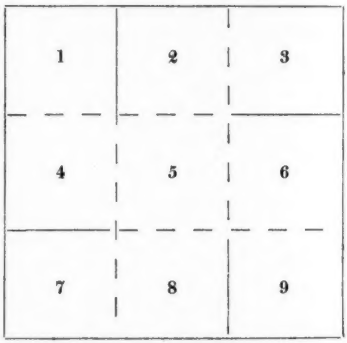
DOUBROW, S. & ROUSSET, J. Sur un procédé de technique histologique concernant la coupe en série des petits Nématodes. Bull. d'Histol. Appl. **6**, 416–417. 1929.

Two difficulties in cutting serial sections thru the chitinous sheaths and heterogeneous contents of Nematodes are overcome by the following technic:

1. Fix in modified Shaudinn's solution ($HgCl_2$ saturated in 80% alcohol with 5% acetic acid) for 4 to 12 hours.
2. Transfer to iodized 80% alcohol for a few days to few weeks, then thru several transfers of 90% (and to 100% if desired) then to butyl alcohol for 24 hours.
3. Transfer to paraffin oven at 56° C. in butyl alcohol saturated with paraffin for 24 hours, and then thru 1 or 2 transfers of pure paraffin, 24 hours each. Embed and stain as usual.

Sections 4 μ thick were thus obtained.

LUCAS, M. S. Method for concentrating and sectioning protozoa. Science, **70**, 482–483. 1929.



Use vial with wide mouth and round bottom or lowest fourth of a test tube. Allow protozoa to settle at bottom before changing fluids by pipetting them off with a wide mouthed pipette to 4 mm. of the level of the protozoan mass. This requires more frequent changes, but in so doing a great loss of specimens is prevented.

Add melted paraffin quickly after removing xylol. Keep under heat bulb whilechanging paraffin. After completing infiltration, pipette off most of the paraffin, leaving enough to cover specimens. Remove vial from under the bulb and let the remaining paraffin solidify on the tip of a large dissecting needle by moving it gently as the mass cools. Use as little paraffin as possible. The protozoa are now temporarily embedded on the needle tip.

Submerge a paper box with the 4 corners open at the bottom, (follow diagram; fold so that 1 would be outside of 2, 3 outside of 6, 9 outside of 8 and 7 outside of 4.), into a watch crystal with melted paraffin under the heat bulb. Lower the temporary block in the box and let it completely melt and fuse with the surrounding paraffin. Allow the protozoa to settle at bottom, and if well concentrated they will fall in one small spot. Specimens with a longer axis are likely to settle with that side against the bottom, securing thus a definite orientation. Lift the box from the crystal and transfer to ice water after the sides have solidified.